DEVELOPMENTAL RESPONSES IN CHICK EMBRYOS EXPOSED TO DIFFERENT GASEOUS ENVIRONMENTS FOR SHORT PERIODS DURING EARLY STAGES OF DEVELOPMENT

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by

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CHAPTER I

INTRODUCTION AND HISTORICAL BACKGROUND

During early stages of development, various parts of an embryo grow and differentiate rapidly in a specific fashion characteristic of that species. During early stages there is not only a rapid increase in the number of cells in the embryo, but also a continued transformation of the cells into different functional units, i.e., tissues and organs. Any factor, either internal or external, interfering with these morphogenetic processes will lead to a deviated pattern of growth and development.

Among the several external factors which can influence the development of an embryo, one of the most important is the amount of oxygen available to rapidly proliferating tissues. It is now generally agreed that both an increase as well as a decrease in the supply of oxygen to the tissues proves detrimental and may lead to the development of various types of malformations in the embryo.[1] Different experimental approaches designed specifically to study the etiology of a deviated pattern of development may help understand the

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[1] L. W. Taylor, and G. O. Kreutziger, "The Gaseous Environment of the Chick Embryo in Relation to Its Development and Hatchability. 3. Effect of Carbon Dioxide and Oxygen Levels During the Period of the Ninth Through the Twelfth Days of Incubation," Poultry Science, VL (September, 1966), 867-884.

mechanisms which render continued life precarious or even altogether impossible.

Interest in the gaseous environment of the chick embryo falls into four primary categories: (1) the effects of pre-incubation factors on subsequent embryogenesis, (2) the effects of pre-incubation factors on hatchability and viability of the chicks, (3) the effects of the gaseous environment during incubation on embryogenesis, and (4) the effects of the gaseous environment during incubation on hatchability and viability of the chicks. Because of the economic aspects of poultry farming, the determination of the optimum gaseous requirements of the developing embryo has received considerable attention from researchers. A knowledge of how much the conditions can be changed without a loss of hatchability or viability of the chicks is also of interest.

As a means of minimizing the metabolic rate of blastodisk cells in fertile chick eggs during storage, eggs are stored at cool temperatures, though not so cold as to be incompatible with life. Cells of the blastodisk carry on metabolic activity at a decreased rate during storage. Fertile eggs may be stored for up to seven days at 10-12° C with little loss of hatchability. After prolonged storage hatchability decreases and incubation time is increased.[1]

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[1]W. A. Becker, "The Storage of White Leghorn Hatching Eggs in Plastic Bags," Poultry Science, XLIII (September, 1964), 1109-1112.

To further decrease the rate of metabolism during storage, Proudfoot, 1964,[1] 1965,[2] 1966,[3] Gowe, 1965,[4] Arora, 1963,[5] and Becker, 1963,[6] and 1964,[7] found that by sealing hatching eggs in plastic bags the storage time could be increased without any loss in hatchability. By flushing the bags with nitrogen before sealing, the storage time can be prolonged still further.[8]

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[1]F. G. Proudfoot, "The Effects of Plastic Packaging And Other Treatments on Hatching Eggs," Canadian Journal of Animal Science, XLIV (April, 1964a), 87-95.

[2]F. G. Proudfoot, "The Effect of Film Permeability And Concentration of Nitrogen, Oxygen, and Helium Gases on Hatching Eggs Stored in Polyethylene and Cryovac Bags," Poultry Science, XLIV (July, 1965), 636-644.

[3]F. G. Proudfoot, "The Use of Sealed Cryovac and Polyethylene Case Liners With and Without Nitrogen Gas For the Preservation of Hatching Eggs," Poultry Science, VL (January, 1966), 105-108.

[4]R. S. Gowe, "On the Hatchability of Chicken Eggs Stored in Plastic Bags Flushed with Nitrogen Gas," Poultry Science, XLIV (May, 1965), 492-495.

[5]K. L. Arora, and I. L. Kosin, "The Effect of Different Gaseous Environments on the Blastoderm in Chicken Eggs Stored at 25±1° C.," Progress Reports, Poultry Council, Washington State University, (1963), 114-119.

[6]W. A. Becker, J. V. Spencer, and J. L. Smartwood, "Storing Hatching Eggs in Plastic Bags," Poultry Science, XLII (December, 1963), 1256.

[7]W. A. Becker, "The Storage of White Leghorn Hatching Eggs in Plastic Bags," Poultry Science, XLIII (September, 1964), 1109-1112.

[8]F. G. Proudfoot, "Note on the Effect of Nitrogen And Other Gases on Hatching Eggs Stored in Plastic Bags," Canadian Journal of Animal Science, XLIV (April, 1964b), 120-121.

Swanson found that the quality of market eggs (those used for eating) subjected to prolonged storage could be improved significantly by storing them in plastic bags flushed with carbon dioxide.[1] Palmer reported an increase in market egg quality when stored in egg cartons lined with plastic compared to those stored in regular cartons.[2] The mode of action of the plastic lining is thought to be the prevention of gaseous exchange between the egg and atmosphere and prevention of water loss from the egg.[3]

When a fertilized chick egg is incubated and starts to develop, the gaseous environment becomes much more important than before incubation. During storage the blastodisk is quiescent, but upon being warmed in the incubator the metabolic rate of the cells is vastly increased as they undergo proliferation and differentiation.

The need of living cells for an adequate supply of oxygen is well known. Of the three major gaseous components of air (oxygen, about 20%, nitrogen, about 78%, and carbon dioxide, about 0.4% or less) oxygen is undoubtedly the most

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[1]M. W. Swanson, "A Proposal for the Use of Carbon Dioxide in Retail Egg Cartons," Poultry Science, XXXII (March, 1953), 369-371.

[2]D. H. Palmer, and W. C. Skoglund, "Maintaining Interior Egg Quality by Holding Shell Eggs in a Case With A Petroleum Resin Liner," Poultry Science, XLIII (January, 1964), 25-30.

[3]Ibid.

important for the maintenance of life.[1] Equally as detrimental as an inadequate supply of oxygen, levels of oxygen much above normal prove to be quite toxic to living systems. The mechanisms of oxygen toxicity are not well understood, but some generalized effects have been noted.

> High oxygen retards growth as oxidations are accelerated, and eventually kills cells. Coordinative processes and cellular organelles are affected. Furthermore, oxygen increases mutation rates--presumably through the action of the peroxides which it generates.[2]

At least six factors must be taken into consideration when the gaseous environment of a developing chick embryo is considered: (1) the stage of development of the embryo when exposed to the gases, (2) the duration of exposure, (3) the relative humidity of the gaseous environment, (4) temperature, (5) the total pressure of the gases, and (6) the partial pressures of the respective gases involved.[3] Humidity is an important factor only during the latter half of incubation, and not during the first half when it has little effect on gaseous exchange or heat conduction.[4]

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[1]Arthur C. Guyton, Textbook of Medical Physiology (Philadelphia: W. B. Saunders Company, 1966), p. 566.

[2]S. M. Siegel, L. Halpern, G. Davis, and C. Giumerro, "The General and Comparative Biology of Experimental Atmospheres and Other Stress Conditions. Oxygen Toxicity In Plant and Animal Forms at One Atmosphere or Less," Aerospace Medicine, XXXIV (November, 1963), 1034-1037.

[3]O. E. Nelsen, "The Effects of Increased Atmospheric Pressures Upon Early Chick Development," Journal of Morphology, XCVI (March, 1955), 359-374.

[4]Ibid.

A low rate of hatchability at high elevations was found by Wilgus to be caused by hypoxia due to the lowered carbon dioxide and oxygen tensions present at higher altitudes.[1] He also obtained data suggesting that the ability of chick embryos to resist hypoxia might be hereditary.[2] Davis, also studying the effects of high altitudes on hatchability, felt that it would be desirable to genetically select for high hatchability rates in lowered oxygen tensions rather than supplement the available oxygen during incubation.[3]

A developmental biologist is for the most part not interested in the economics of a high rate of hatchability, but in the responses of a developing embryo to less than optimum environmental conditions. Studying abnormal embryological development, or teratogenesis, leads to better understanding of normal development.

Chick embryos are appropriate to the study of teratogenesis because they are easily obtained, economical, easy to maintain and handle, and are free from maternal influence.

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[1]G. T. Davis, "Influence of Oxygen Concentration on Hatchability and on Selecting for Hatchability," Poultry Science, XXXIV (January, 1955), 107-113.

[2]H. S. Wilgus, and W. W. Sadler, "Incubation Factors Affecting Hatchability of Poultry Eggs. 1. Levels of Oxygen And Carbon Dioxide at High Altitude," Poultry Science, XXXIII (May, 1954), 460-471.

[3]Ibid.

Three ranges of response to abnormal gaseous environments may be noted: (1) a latent range in which there is no apparent effect on the embryo, (2) a teratogenic range, and (3) a lethal range.[1] In general, tolerance of embryonic tissues for abnormal proportions of oxygen, nitrogen, and carbon dioxide decreases as growth progresses. The lethal range increases in breadth, while the latent and teratogenic ranges decrease in the later phases of incubation.[2]

Responses of chick embryos to abnormal environmental conditions may be generalized as taking place in other vertebrate species, including man. Using chick embryos Allen found "that abnormally high partial pressures of oxygen exert their effects through an influence on the vascular system in higher organisms."[3] Normal to low oxygen tensions appear to initiate vascularization; blood vessels are formed in response to a concomitant demand of tissues for oxygen. Premature infants kept in incubators with a high partial pressure of oxygen often develop retrolental fibroplasia. a condition in which choroid blood vessels atrophy or fail to develop.

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[1]C. T. Grabowski, and J. A. Parr, "The Teratogenic Effects of Graded Doses of Hypoxia on the Chick Embryo," American Journal of Anatomy, CIII (November, 1958), 313-347.

[2]Ibid.

[3]S. C. Allen, "A Comparison of the Effects of Nitrogen Lack and Hyperoxia on the Vascular Development of the Chick Embryo," Aerospace Medicine, XXXIV (October, 1963), 897-899.

> In such cases the retinal vessels fail to develop and the children are blind. The dimensions of the infant eye-ball are such that the oxygen could diffuse readily into the level of the intraocular vascular system. Under these circumstances the oxygen requirements could be met without the intervention of a vascular system which would fail to develop or regress in the absence of an hypoxic stimulus for capillarization.[1]

This same general effect of the failure of oxygen transport mechanisms to develop when sufficient diffusion occurs to meet the oxygen demands of tissues was noted by Taylor. He states that "the vascularity of the allantois, as well as the erythrocyte count and the hemoglobin concentration are significantly decreased after exposure to high O_2 levels."[2] Besides exposure to high oxygen levels, retarded growth of the allantoic blood vessels may occur in the absence of turning of the egg during incubation.[3] Exposure of humans to low oxygen levels for a sufficient length of time may stimulate hematopoiesis and promote an increased vascularity of tissues.[4] In chick embryos "hematopoiesis is not stimulated by exposure to low O_2 until after the 15th day of incubation."[5]

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[1] Ibid.

[2] Taylor, loc. cit.

[3] A. L. Romanoff, The Avian Embryo (New York: Macmillan, 1960), p. 1134.

[4] Guyton, op. cit., p. 625.

[5] Taylor, loc. cit.

A possible explanation for this is offered by Needham. Primitive red blood cells present in the embryo during the first half of incubation arise from the blood islands of the yolk sac. A different type of haemoglobin originating in the bone marrow of the embryo appears in the second half of the incubation period.[1]

There are many ways of producing abnormalities in chick embryos. As Nelsen observed, "whether induced by hereditary influences or by environmental conditions, anomalisms often are associated with retarded development in various degrees."[2] In either case, depressed metabolic mechanisms are a likely cause of retardation. Abnormalities may be caused by retarding the normal developmental process in some way. In experiments carried out at Drake University in 1968, Arora stored fertile eggs at room temperature for seven days and longer. When incubated, the embryos exhibited a high rate of mortality and retarded development. Ancel coated the shells of hatching eggs with a substance impervious to the passage of gases to produce varying degrees of hypoxia.[3]

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[1]Joseph Needham, Biochemistry and Morphogenesis, (London: Cambridge University Press, 1942), p. 595.

[2]O. E. Nelsen, "Hypoxia and Maldevelopment of Early Alarplate Tissue in Mid- and Fore-Brain Regions of the Chick Embryo," Growth XXIV (December, 1960), 361-383.

[3]P. Ancel, "Recherche sur les Relations Entre L'Effet Letal et L'Effet Teratogene do la Carence en Oxygene Chez L'Embryon du Poule. (An Investigation of the Relationship Between Lethal and Teratological Effects of Oxygen Deficiency In the Chick Embryo)." Acta Anatomy, XXXVIII (January, 1959), 147-159.

In other experiments he subjected chick embryos to short periods of cooling at various times during development.[1] Nelsen produced hypoxia by controlling the proportions of nitrogen and carbon dioxide during early incubation.[2] He also studied the effects of hyperbaric pressures on chick embryogenesis.[3] Grabowski produced hypoxia by incubating eggs in lowered atmospheric pressures for different lengths of time at incubation periods ranging from eighteen hours to nine days.[4]

The importance of nitrogen and carbon dioxide in normal embryogenesis was realized more recently than was the importance of oxygen. Volskii found that chick embryos show a progressive gain in total egg nitrogen content from the time of laying to the time of hatching.[5]

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[1] P. Ancel, "Recherches sur les Malformations Determinees Par le Refroidissement Temporaire de l'Oef de Poule et Compatibles avec la Vie des Poussins. (A Study of Chicken Livability and of Malformation Produced by Temporary Cooling of Chicken Eggs)," J. Embryol. Exp. Morph. VII (September, 1959), 330-334.

[2] O. E. Nelsen, "Hypoxia and Maldevelopment of Early Alarplate Tissue in Mid- and Fore-Brain Regions of the Chick Embryo," Growth XXIV (December, 1960), 361-383.

[3] O. E. Nelsen, "The Effects of Increased Atmospheric Pressures Upon Early Chick Development," Journal of Morphology, XCVI (March, 1955), 359-374.

[4] C. T. Grabowski, "Lactic Acid Accumulation as a Cause of Hypoxia-Induced Malformations in the Chick Embryo," Science, CXXXIV (October, 1961), 1359-1360.

[5] M. I. Volskii, "The Assimilation of Nitrogen by Animal Organisms as Exemplified by Chicken Embryos and Honeybee Pupae," Doklady, Biol. Sci. Sec., Nos. 1-6, AIBS Translation (May, 1960), 895.

Allen reported that four day chick embryos failed to develop further when incubated in an atmosphere devoid of nitrogen but otherwise normal.[1] His method was to reduce the total air pressure of the incubating atmosphere, but maintain a partial pressure of oxygen equivalent to that at sea level by adding supplemental oxygen. Boriskin obtained similar results by incubating eggs in an atmosphere at normal pressure in which the nitrogen was replaced by helium.[2]

Taylor obtained data showing that a concentration of about 0.4% carbon dioxide stimulates embryonic development; he found a consistent, though not statistically significant increase in hatchability at this level of carbon dioxide.[3] Carbon dioxide present in the incubating atmosphere in concentrations greater than 10% is lethal in the first 96 hours of incubation.[4] Spratt stated that "It is possible that carbon dioxide may be as fundamental a requirement of

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[1]Allen, loc. cit.

[2]V. V. Boriskin, P. V. Oblapenko, V. V. Rol'nik, and B. M. Savin, "Developmental Potentialities of the Animal Organism When Atmospheric Nitrogen is Replaced by Helium," Akademiya nauk SSSR Doklady, CXLIII (March, 1962), 475-478.

[3]L. W. Taylor, R. A. Sjodin, and C. A. Gunns, "The Gaseous Environment of the Chick Embryo in Relation to Its Development and Hatchability. 1. Effect of Carbon Dioxide And Oxygen Levels During the First Four Days of Incubation On Hatchability," Poultry Science, XXXV (November, 1956), 1206-1215.

[4]Ibid.

the embryo as oxygen."[1] He reported that partial removal of carbon dioxide inhibited differentiation and morphogenesis of the central nervous system.[2]

One effect of carbon dioxide in the egg is to decrease the pH of the albumin as carbonic acid is formed.[3] Sadler postulated that a low pH in the albumin prevents premature breakdown of the chalaziferous membrane.[4] This membrane is necessary to separate the yolk from the albumin; its breakdown normally occurs after the amnion is completely formed.[5]

Nitrogen, as the largest component of air is important in normal development for the maintenance of pressure. In hypobaric experiments Nelsen found that:

> Even though the O_2 level is sufficiently high for normal development under ordinary pressure conditions, a decrease in pressure by the removal of N_2 below a given level results in maldevelopment.[6]

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[1] N. T. Spratt, "Carbon Dioxide Requirements of the Early Chick Embryo," Anatomical Record, CV (May, 1949), 598.

[2] Ibid.

[3] L. W. Taylor, and G. O. Kreutziger, "The Gaseous Environment of the Chick Embryo in Relation to Its Development and Hatchability. 3. Effect of Carbon Dioxide and Oxygen Levels During the Period of the Ninth Through the Twelfth Days of Incubation," Poultry Science, VL (September, 1966), 867-884.

W. W. Sadler, "Chronological Relationship of the Disappearance of the Vitelline Membrane and the Closure of The Amnio-chorion in Avian Embryos and Its Implications," Poultry Science, XXXIV (June, 1955), 754-760.

[5] Ibid.

[6] O. E. Nelson, "The Effects of Increased Atmospheric Pressures Upon Early Chick Development," Journal of Morphology, XCVI (March, 1955), 359-374.

Nitrogen used to produce hypoxia by displacement of oxygen at normal pressures "is equally as potent in producing anomalies and death as that produced by vacuum."[1]

Early investigators assumed that the various gases present in the incubating atmosphere exerted their effects independently. Evidence has accumulated to show that the effects of gases are interrelated. There may also be a relationship between gaseous concentrations and incubating temperature. Nelsen reported an increased tolerance to high oxygen pressures when the temperature was lowered slightly from the normal 38° C.[2] He also found the toxic effects of oxygen to be antagonized by the presence of nitrogen.[3]

When carbon dioxide increases, it is usually in response to a lowered level of oxygen. Additive and complimentary effects were noted by Taylor. Levels of carbon dioxide above normal cause vasoconstriction of yolk sac blood vessels and possibly of chorio-allantoic vessels.[4] Nelson cites several examples of the vasoconstrictive

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[1] O. E. Nelsen, "The Effects of Increased Atmospheric Pressures Upon Early Chick Development," Journal of Morphology, XCVI (March, 1955), 359-374.

[2] Ibid.

[3] C. T. Grabowski, and J. A. Parr, "The Teratogenic Effects of Graded Doses of Hypoxia on the Chick Embryo," American Journal of Anatomy, CIII (November, 1958), 313-347.

[4] L. W. Taylor, and G. O. Kreutziger, "The Gaseous Environment of the Chick Embryo in Relation to Its Development and Hatchability. 3. Effect of Carbon Dioxide and Oxygen Levels During the Period of the Ninth Through the Twelfth Days of Incubation," Poultry Science, VL (September, 1966), 867-884.

effects of high concentrations of oxygen on humans.[1]

Although it appears that the primary effect of hypoxia is the alteration or disruption of enzyme systems, Grabowski presented evidence that embryonic damage may be mechanical. Accumulation of lactic acid during extended anaerobiosis causes "a tremendous edema (embryo volume increases up to tenfold) which persists for several hours."[2] Numerous blisters and hematomas cause maldevelopment of adjacent structures.[3]

Most investigators thus far have varied the proportions of gases in the incubating environment by controlling the pressure within the incubating chamber or by flowing a known mixture of gases over the eggs while they are incubating at atmospheric pressure. High and low concentrations of gases have been tried, but no mention has been made of the effects of pure gaseous environments on developing chicks.

This investigation was designed to study the effects of atmospheres of 100% nitrogen (total anoxia), 100% oxygen, and 100% carbon dioxide on the developing chick embryo. Exposure to the gases was begun at the time incubation was started, and lasted for periods ranging up to 32 hours.

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[1]O. E. Nelsen, "The Effects of Increased Atmospheric Pressures Upon Early Chick Development," Journal of Morphology, XCVI (March, 1955), 359-374.

[2]C. T. Grabowski, "Lactic Acid Accumulation as a Cause of Hypoxia-Induced Malformations in the Chick Embryo," Science CXXXIV (October, 1961), 1359-1360.

[3]Ibid.

CHAPTER II

METHODS AND MATERIALS

The effects of exposure to different pure gaseous environments (100% oxygen, 100% nitrogen, and 100% carbon dioxide) on the early embryonic development of chick embryos were studied.

Fertilized eggs of White Leghorn (Gallus domesticus) hens were obtained from the Hy-Line Poultry Farm, Johnston, Iowa. Eggs from flock number RB 256 were used for all experiments. During the period of experimentation, eggs were delivered to Hy-Line by the supplier on Mondays and Thursdays. Eggs for experiments were picked up at the Hy-Line Poultry Farm on Monday, Thursday, and Friday afternoons. Eight dozen eggs were obtained in cardboard cartons of one dozen eggs for each complete experiment. The eggs were divided into four groups of two dozen eggs. Three of the groups received an experimental treatment and one group served as controls. The tops of all cartons were removed to allow adequate air circulation around the eggs which were left in the cartons.

Cryovac plastic bags (polyvinylidene chloride copolymer, type 930 standard, Manufactured by the W. R. Grace Company, Cryovac Division, P. O. Box 464, Duncan, South Carolina 29334) were used to contain the eggs and atmospheres. Oxygen, nitrogen, and carbon dioxide gases were obtained

from R. and R. Welding Supply Company, 1700 Second Avenue, Des Moines, Iowa.

A series of 25 preliminary experiments was performed in order to determine the general tolerance of the embryos for the various pure gaseous environments, and to establish the best exposure times for a comparative study. The duration of exposure ranged from 8 to 32 hours, and the period of incubation at which exposure was made ranged from one week before incubation to five days after the start of incubation. Embryos exhibited the greatest tolerance for oxygen, and the least tolerance for carbon dioxide. There was a marked decrease in tolerance to all gases when application was made in the later phases of incubation. Since tolerance is greatest at the beginning of incubation, this was taken as a starting point for the application of gases in all subsequent experiments.

Two dozen eggs were placed in each Cryovac bag after each egg was coded for the treatment to be given. The bags were inflated using a length of tygon tubing connected directly to the tank of gas without a regulator. After slowly inflating the bags and collapsing them two times, they were inflated and the necks sealed by tying with string.

The bags containing the eggs were placed in a David Bradley electric cabinet forced air incubator at a temperature of 38° C and about 85% relative humidity. The two dozen

control eggs were incubated in the normal incubator atmosphere in cardboard cartons with the tops removed; Cryovac bags were not used for any of the control groups. No warm up period was provided for the eggs; the incubation times given are the actual times that the eggs were in the incubator. At the end of the desired gaseous exposure time for each group, the eggs were removed from the Cryovac bag containing the experimental atmosphere and returned to the incubator while still in the egg cartons. When the last experimental group of eggs was removed from the Cryovac bag, all eight dozen eggs were removed from the egg cartons and put in conventional incubator trays. For all runs, exposure to the various gases began at the time incubation was started. Table I summarizes the treatments given to each of the four groups of eggs in the nine runs which comprise this study.

After 38 hours total incubation time, (48 hours for runs 3 and 4) one dozen eggs from each group was removed from the incubator for examination. The shell surrounding the air space in the large end of each egg was removed with forceps. The inner shell membrane could usually be peeled from the underlying yolk, but sometimes it adhered to the vitelline membrane. A small amount of physiological saline (0.9% NaCl in distilled water) was spread on the inner shell membrane allowing it to be removed without damage to the vitelline membrane.

TABLE I

SUMMARY OF EXPERIMENTAL TREATMENTS*

Run Number	Group 1**	Group 2**	Group 3**	Group 4**
1	Control	16 hrs. N_2	24 hrs. N_2	32 hrs. N_2
2	Control	16 hrs. N_2	24 hrs. N_2	32 hrs. N_2
3***	Control	16 hrs. N_2	24 hrs. N_2	32 hrs. N_2
4***	Control	16 hrs. N_2	24 hrs. N_2	32 hrs. N_2
5	Control	10 hrs. O_2	10 hrs. N_2	10 hrs. CO_2
6	Control	5 hrs. O_2	5 hrs. N_2	5 hrs. CO_2
7	Control	16 hrs. O_2	16 hrs. N_2	16 hrs. CO_2
8	Control	16 hrs. Air	24 hrs. Air	32 hrs. Air
9	Control	24 hrs. O_2	24 hrs. N_2	24 hrs. CO_2

*All treatments were started at the beginning of incubation.

**24 eggs in each group; 12 eggs were examined after 38 hours incubation, the remainder were examined after 7 days.

***12 eggs were examined after 48 hours incubation, the remainder were examined after 7 days.

The yolk normally rotated so the embryo was uppermost, but this did not always occur. Frequently, the albumin had to be drained from the shell until the yolk was gently flattened in the small end of the shell. The yolk could usually be rotated until the embryo was on top by gentle prodding with the handle of the forceps. Sometimes the vitelline membrane was so weakened by the experimental

treatment that the weight of the yolk when the albumin was removed caused the membrane to rupture.

With the vitelline membrane intact and the blastodisk uppermost, the embryo was then stained using agar impregnated with neutral red stain. The following method for preparing stain impregnated agar slides was obtained from Dr. Kashmiri L. Arora of the Drake University biology staff.

1. Prepare 1%-2% solution of agar in distilled water.
2. Boil breifly until the agar is completely dissolved.
3. While it is still warm, pour a film of the agar solution on cleaned microscope slides. Avoid entrapping air bubbles. An eye dropper may be used to flood the slide with as much solution as possible, as long as it does not run over the edge.
4. Allow the slides to dry thoroughly for one or more days in a dust free place.
5. Place the dried slides with agar film in a Coplin jar filled with a 1% solution of neutral red or Nile blue sulphate stain and let them stand for one or more days.
6. Wash off the excessive dye and allow the agar to dry again. These slides may be kept indefinately in a dust free wrapping.
7. To use the slides, soak them for a few minutes in water or the solution used to stain them. Cut squares of agar and lift them from the slide with a razor blade.

To stain the embryo a 5-10 mm. square of agar is placed directly on the embryo with a pair of forceps. Using an eye dropper and physiological saline, the agar is kept moist to prevent it from sticking to the vitelline membrane. Forceps are used to remove the agar after 5-10 minutes.

The embryo was then examined using a Bausch & Lomb Stereo-Zoom dissecting microscope (Bausch & Lomb Incorporated, 635 St. Paul Street, Rochester 2, New York 14602.) For illumination a Bausch & Lomb variable intensity Nicholas microscope illuminator (model 31-33-53) was used. The embryo was kept moist with saline solution to prevent the delicate tissues from drying. An additional 10-20 minute waiting period after removal of the agar block from the embryo allows the stain to localize in the embryonic tissues. Since neutral red is a vital stain, the embryonic tissues are not harmed by the stain and the heart may continue to beat for an hour or more after removal from the incubator if the tissues are kept moist. Forceps may be used to gently peel the vitelline membrane from the embryo to allow clearer viewing, but this is not necessary.

All embryos were examined for gross malformations, and the number of somites was recorded for each embryo. The length of each embryo and the width of the area vasculosa was measured under the dissecting microscope using vernier calipers.

Photographs of abnormal or representative embryos from each group were taken using a Honeywell Pentax Spot-matic single lens reflex 35 mm. camera fitted with a 50 mm. f/1.4 lens on a bellows. The camera was mounted on a Polaroid copy stand; the eggs were left in the egg cartons

or were placed in individual egg holders while being photographed.

The remaining eggs were examined after one week total incubation time. The embryos were removed from the shell and placed in a dish of physiological saline. After freeing the embryos of all extraembryonic membranes, they were drained on a paper towel, placed on a 1 in. x 1 in. square of newspaper, and weighed individually on a triple beam balance after first calibrating the balance with a square of newspaper.

Representative and abnormal embryos were fixed in Bouin's fixative.[1] Photographs were taken after fixation using the photographic equipment described above.

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[1]Roberts Rugh, Experimental Embryology (Minneapolis: Burgess Publishing Company, 1965), p. 16.

CHAPTER III

RESULTS AND INTERPRETATION OF DATA

The response of developing chick embryos to exposure to various pure gaseous environments was studied. Fertilized eggs of White Leghorn hens were incubated in Cryovac plastic bags inflated with 100% nitrogen, 100% oxygen, or 100% carbon dioxide for the first 5, 10, 16, and 24 hours of incubation, and to 100% nitrogen for the first 32 hours of incubation. Half of the eggs in each group were examined after 38 hours total incubation time, and the other half were examined after 7 days incubation. Measurements of embryo length, width of area vasculosa, and number of somites were made on the embryos examined after 38 hours incubation. Embryos were weighed and examined for gross developmental malformations after 7 days of incubation.

Table II gives mean values plus or minus one standard deviation for each of the measurements for each run, and gives t values for each measurement as compared to the appropriate control measurement for the same run.[1] Embryos in the experimental groups of runs 1 and 2 showed a significant ($P < 0.01$) retardation in development, the degree of

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[1] A. Lawrence O'Toole, Elementary Practical Statistics (New York: The Macmillan Company, 1964), pp. 216-219.

TABLE II

MEAN VALUES FOR CONTROLS AND EXPERIMENTALS
AND ASSOCIATED T VALUES*

		38 HOURS INCUBATION			7 DAYS INCUBATION	
Run Number	Treatment	N	Embryo Length (mm.)	Area Vasculosa (mm.)	Somite Number	N	Weight at 7 Days (gms.)
1	Control	10	4.08 ± .430	6.23 ± .853	9.6 ± 2.16	9	.762 ± .051
1	16 hrs. N_2	12	1.88 ± .457 t = 3.67	1.97 ± .673 t = 40.19	- - - - -	6	.623 ± .070 t = 4.09
1	24 hrs. N_2	6	- - - - - -	- - - - - -	- - - - -	4	.478 ± .029 t = 12.74
1	32 hrs. N_2	5	- - - - - -	- - - - - -	- - - - -	2	.285 ± .050 t = 12.86
2	Control	11	4.74 ± .797	7.65 ± 1.39	12.9 ± 2.34	11	.683 ± .022
2	16 hrs. N_2	11	2.86 ± .312 t = 7.32	4.73 ± .681 t = 19.73	5.4 ± 3.06 t = 6.47	8	.576 ± .037 t = 7.54
2	24 hrs. N_2	5	1.82 ± .150 t = 11.77	3.20 ± .656 t = 27.13	- - - - -	7	.420 ± .089 t = 7.65
2	32 hrs. N_2	10	- - - - - -	- - - - - -	- - - - -	3	.250 ± .052 t = 12.74
5	Control	9	5.16 ± .755	7.96 ± .892	14.8 ± 2.96	11	.786 ± .017

TABLE II (Continued)

Run Number	Treatment	N	Embryo Length (mm.)	Area Vasculosa (mm.)	Somite Number	N	Weight at 7 days (gms.)
5	10 hrs. CO_2	12	3.01 ± .860 t = 6.07	3.43 ± 1.07 t = 10.53	3.42 ± 2.50 t = 9.33	12	.630 ± .110 t = 4.94
5	10 hrs. N_2	11	3.95 ± 1.51 t = 2.33	5.03 ± 1.22 t = 6.12	10.5 ± 2.81 t = 3.31	10	.684 ± .080 t = 3.95
5	10 hrs. O_2	12	4.30 ± .163 t = 1.06	6.33 ± 1.29 t = 3.42	11.8 ± 1.25 t = 2.90	11	.710 ± .067 t = 3.64
6	Control	12	4.62 ± .906	7.61 ± 1.96	13.0 ± 3.42	8	.690 ± .019
6	5 hrs. CO_2	9	3.06 ± .458 t = 4.73	4.59 ± .890 t = 4.72	5.9 ± 2.32 t = 5.63	10	.615 ± .082 t = 2.81
6	5 hrs. N_2	12	3.89 ± .159 t = 2.79	6.20 ± .170 t = 2.49	10.2 ± 2.49 t = 2.30	8	.685 ± .015 t = .584
6	5 hrs. O_2	11	3.84 ± .179 t = 2.92	6.50 ± .190 t = 1.95	10.4 ± 1.79 t = 2.30	9	.731 ± .068 t = 1.74
7	Control	9	4.73 ± .650	7.48 ± 1.77	13.6 ± 2.81	11	.732 ± .072
7	16 hrs. CO_2	12	- - - - - -	- - - - - -	- - - - - -	11	.446 ± .019 t = 12.71
7	16 hrs. N_2	10	3.31 ± .503 t = 5.30	4.52 ± .952 t = 4.46	4.9 ± 2.97 t = 7.22	4	.660 ± .050 t = 2.18

TABLE II (Continued)

Run Number	Treatment	N	Embryo Length (mm.)	Area Vasculosa (mm.)	Somite Number	N	Weight at 7 Days (gms.)
7	16 hrs. O_2	12	3.54 ± .760 t = 3.13	5.23 ± .600 t = 3.65	8.3 ± .775 t = 23.48	11	.651 ± .026 t = 2.45
8	Control	12	4.83 ± .258	7.23 ± .656	13.9 ± 1.73	10	.789 ± .070
8	16 hrs. Air	12	4.37 ± .454 t = 3.03	6.12 ± .287 t = 5.36	11.5 ± 1.55 t = 3.61	10	.757 ± .074 t = 1.01
8	24 hrs. Air	12	4.33 ± .129 t = 6.00	6.14 ± .868 t = 3.47	10.7 ± 1.21 t = 5.34	12	.670 ± .055 t = 4.38
8	32 hrs. Air	11	3.86 ± .114 t = 11.80	5.77 ± .265 t = 7.12	8.6 ± 2.53 t = 5.79	9	.698 ± .081 t = 2.61
9	Control	11	4.51 ± .528	7.10 ± .872	11.8 ± 1.84	11	.782 ± .064
9	24 hrs. CO_2	12	- - - - - -	- - - - - -	- - - - - -	11	.299 ± .033 t = 13.05
9	24 hrs. N_2	5	2.54 ± .950	3.52 ± 1.42	- - - - - -	6	.512 ± .072 t = 7.67
9	24 hrs. O_2	12	4.60 ± .760 t = .332	5.70 ± .832 t = 3.91	9.3 ± 1.54 t= 3.52	9	.737 ± .042 t = 1.88

*Mean ± 1 standard deviation. T values computed for each experimental measurement compared to the appropriate control value for that run.

retardation being greatest in those embryos exposed to nitrogen for the longest period of time. In runs 5 and 6 the carbon dioxide groups showed significant retardation ($P < 0.05$) and the corresponding groups in runs 7 and 9 were so severely retarded that they were just beginning primitive streak formation. The nitrogen groups of runs 5, 6, 7, and 9 all showed significant retardation ($P < 0.05$) in measurements made at 38 hours of incubation. The weights of the embryos in the nitrogen groups were significantly less ($P < 0.05$) except in the group exposed for 5 hours in which the retardation was not statistically significant. Exposure to oxygen for 5 hours produced a significant decrease ($P < 0.05$) in embryo length and somite number, but not in area vasculosa width. There was an insignificant increase ($P < 0.10$) in embryo weight after 7 days of incubation. Ten and 16 hours exposure to oxygen produced significant ($P < 0.05$) decreases in all measurements except embryo length in the group exposed for 10 hours. Exposure to oxygen for 24 hours gave a significant decrease ($P < 0.05$) in area vasculosa width and somite number, an insignificant increase in embryo length, and an insignificant decrease in weight after 7 days incubation.

Ideally, there should be a difference of only one variable between control and experimental groups. The variable in these experiments was the gaseous environment to which the embryos were exposed during early incubation.

Since eggs in the experimental groups were enclosed in plastic bags and those in the control groups were not, this added another variable. An experiment (run 8) was performed to determine if enclosure in plastic bags filled with compressed air would have a retarding effect on the developing embryos. These data are presented in Table II. Although all surviving embryos were morphologically normal, there was a significant ($P < 0.05$) decrease in all measurements except the weight of embryos exposed to compressed air for the shortest period of time.

The retardation exhibited may have been caused by an increased carbon dioxide concentration within the sealed bags due to metabolic waste products of the developing embryos, although this hypothesis was not tested. Ramm found a direct relationship between "the degree of retardation, decrease in developmental stage achieved, and the oxygen tension of the environment."[1] It is also conceivable that the oxygen and carbon dioxide have additive or synergistic effects.

Table III gives the same information as Table II for runs 3 and 4. These runs were duplicates of runs 1 and 2, except that the eggs were examined after 48 hours of incubation rather than 38 hours as in all other runs.

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[1]G. M. Ramm, and J. LaBlanc, "Development of Chick Embryos in Lowered Oxygen Atmospheres," American Zoologist, IV (March, 1964), 322.

TABLE III

MEAN VALUES FOR CONTROLS AND EXPERIMENTALS
AND ASSOCIATED T VALUES*

		48 HOURS INCUBATION			7 DAYS INCUBATION	
Run Number	Treatment	N	Embryo Length (mm.)	Area Vasculosa (mm.)	Somite Number	N	Weight at 7 Days (gms.)
3	Control	10	6.78 ± .932	11.53 ± 1.38	23.1 ± 2.56	11	.658 ± .080
3	16 hrs. N_2	12	3.91 ± 1.35 t = 5.78	8.50 ± 1.78 t = 4.50	11.8 ± 5.22 t = 6.64	4	.668 ± .103 t = .180
3	24 hrs. N_2	12	3.05 ± .500 t = 11.37	5.88 ± 1.55 t = 9.05	7.5 ± 2.28 t = 15.1	6	.461 ± .091 t = 4.40
3	32 hrs. N_2	10	2.53 ± .836 t = 10.44	3.67 ± 1.50 t = 12.22	- - - - - -	7	.296 ± .073 t = 9.67
4	Control	11	6.40 ± .643	12.05 ± 1.15	23.2 ± 2.45	9	.781 ± .052
4	16 hrs. N_2	12	5.76 ± .605 t = 2.45	9.92 ± 1.39 t = 4.02	19.1 ± 1.09 t = 5.06	8	.696 ± .046 t = 3.47
4	24 hrs. N_2	9	3.34 ± .667 t = 8.29	5.98 ± 1.06 t = 12.47	10.1 ± 1.28 t = 15.27	7	.651 ± .070 t = 4.11
4	32 hrs. N_2	5	2.54 ± .442 t = 10.8	4.50 ± 2.17 t = 7.12	- - - - - -	3	.457 ± .013 t = 17.14

*Mean ± 1 standard deviation. T values computed for each experimental measurement compared to the appropriate control value for that run.

A significant decrease ($P < 0.05$) is shown in all measurments except embryo weight in the group exposed to nitrogen for 16 hours, where the decrease is not statistically significant.

It was noted when the eggs were removed from the Cryovac bags that the relative humidity within the bag was always higher than the humidity in the incubator, which is normally about 85%. An experiment was performed to determine if the increased humidity was a factor in retarded embryogenesis. One dozen eggs were incubated in each of two Cryovac bags inflated with compressed air for 32 hours, the maximum time used in any of the runs. In one bag was placed a petri dish containing silica gel to absorb moisture. In the other bag the humidity was allowed to increase as previously noted. The results are shown in Table IV. There was no significant difference ($P < 0.05$) between the two groups in embryo length, width of area vasculosa, or number of somites.

TABLE IV

MEAN AND T VALUES FOR EGGS INCUBATED IN COMPRESSED AIR WITH AND WITHOUT SILICA GEL

Treatment	N	Embryo Length (mm.)	Area Vasculosa (mm.)	Somite Number
Without Silica Gel	8	4.54 ± .642	6.78 ± .593	11.4 ± 1.46
With Silica Gel	9	4.28 ± .690 t = .805	7.42 ± 1.14 t = 1.48	12.0 ± 1.94 t = .726

Table V was prepared using the data in Table II. Eggs exposed to nitrogen, oxygen, and carbon dioxide for 16 and 24 hours were compared to those exposed to compressed air for the same periods of time. It can be seen from the new t values that a significant ($P < 0.05$) retardation occurred in nearly all cases. The exceptions are in the oxygen group of run 9. The retardation of the area vasculosa width is not significant, and a significant ($P < 0.05$) increase in weight at 7 days of incubation is shown.

Table VI gives the percentage of abnormalities and mortalities at 38 hours and 7 days of incubation for the various treatments for all runs. More abnormalities appeared at 38 hours than at 7 days, according to the data. There are several reasons for this. At 38 hours the embryos are not morphologically complex, and abnormalities are easy to detect. Most embryos abnormal at 38 hours died before 7 days. Only gross external abnormalities were detected at 7 days; there were undoubtedly many abnormalities occurring which were not detected. Live embryos not exhibiting gross abnormalities at 38 hours and 7 days were considered to be normal, even though they were frequently severly retarded.

A number of generalized effects of the various gases were observed at the 38 hour examination which are not reflected in the data. Nitrogen exposure (anoxia) disrupted the integrity of the vitelline membrane. The effect became

TABLE V

MEAN VALUES AND T VALUES FOR EXPERIMENTALS
COMPARED TO 16 AND 24 HOURS
IN COMPRESSED AIR

Run Number	Treatment	N	Embryo Length (mm.)	Area Vasculosa (mm.)	Somite Number	N	Weight at 7 Days (gms.)
8	16 hrs. Air	12	4.37 ± .454	6.12 ± .287	11.5 ± 1.55	12	.757 ± .074
1	16 hrs. N_2	12	1.88 ± .457 t = 13.38	1.97 ± .673 t = 19.58	- - - - - - -	6	.623 ± .070 t = 5.40
2	16 hrs. N_2	11	2.86 ± .312 t = 4.66	4.73 ± .681 t = 6.29	5.4 ± 3.06 t = 6.04	8	.576 ± .037 t = 7.24
7	16 hrs. N_2	10	3.31 ± .503 t = 5.05	4.52 ± .952 t = 5.11	4.9 ± 2.97 t = 6.35	4	.660 ± .050 t = 2.86
7	16 hrs. CO_2	12	- - - - - - -	- - - - - - -	- - - - - - -	11	.446 ± .019 t = 14.01
7	16 hrs. O_2	12	3.54 ± .760 t = 3.24	5.23 ± .600 t = 4.59	8.3 ± .775 t = 6.40	11	.651 ± .026 t = 10.29
8	24 hrs. Air	12	4.33 ± .129	6.14 ± .868	10.7 ± 1.21	12	.670 ± .055
1	24 hrs. N_2	6	- - - - - - -	- - - - - - -	- - - - - - -	4	.478 ± .029 t = 8.81
2	24 hrs. N_2	5	1.82 ± .150 t = 32.72	3.20 ± .656 t = 7.57	- - - - - - -	7	.420 ± .089 t = 6.65
9	24 hrs. N_2	5	2.54 ± .950 t = 4.19	3.52 ± 1.42 t = 3.84	- - - - - - -	6	.512 ± .072 t = 4.74
9	24 hrs. CO_2	12	- - - - - - -	- - - - - - -	- - - - - - -	11	.299 ± .033 t = 10.51
9	24 hrs. O_2	12	4.60 ± .760 t = 3.84	5.70 ± .832 t = 1.27	9.3 ± 1.54 t = 2.42	9	.737 ± .042 t = 3.18

TABLE VI

PERCENTAGE OF ABNORMALITIES AND MORTALITIES

Run Number	Treatment	% Abnormalities at 38 Hours	% Mortality at 38 Hours	% Abnormalities at 7 Days	% Mortality at 7 Days
1	Control	8	17	0	25
1	16 hrs. N_2	8	0	0	50
1	24 hrs. N_2	0	58	0	67
1	32 hrs. N_2	0	58	0	83
2	Control	0	8	0	8
2	16 hrs. N_2	25	17	0	33
2	24 hrs. N_2	0	50	0	42
2	32 hrs. N_2	0	25	0	75
3	Control	0	17	0	8
3	16 hrs. N_2	17	50	0	58
3	24 hrs. N_2	17	0	8	42
3	32 hrs. N_2	8	58	0	42
4	Control	0	8	0	25
4	16 hrs. N_2	17	0	0	33
4	24 hrs. N_2	25	25	8	33
4	32 hrs. N_2	17	42	0	75
5	Control	0	25	0	0
5	10 hrs. N_2	8	17	0	17
5	10 hrs. CO_2	0	0	0	0
5	10 hrs. O_2	0	0	0	8
6	Control	8	8	8	33
6	5 hrs. N_2	0	0	17	17
6	5 hrs. CO_2	8	17	0	17
6	5 hrs. O_2	8	8	8	17
7	Control	0	33	0	8
7	16 hrs. N_2	25	17	8	58
7	16 hrs. CO_2	0	0	0	8
7	16 hrs. O_2	17	0	0	8
8	Control	0	0	0	17
8	16 hrs. Air	0	0	8	17
8	24 hrs. Air	0	0	0	0
8	32 hrs. Air	0	8	0	25
9	Control	0	8	0	8
9	24 hrs. N_2	0	25	17	50
9	24 hrs. CO_2	0	0	50	83
9	24 hrs. O_2	17	0	0	0

more pronounced as exposure time was increased. The membrane was often so fragile that it would not support the weight of the agar used to stain the embryo. The weakest area of the vitelline membrane was in the region of the embryo; a raised bulge with the embryo on top could usually be seen on the surface of the yolk. The yolk is normally weighted so that as the egg is turned, the yolk rotates so the blastodisk always comes to the top. This, along with the weakened state of the vitelline membrane made the embryo difficult to position for staining. It was not uncommon for the membrane to bleb or even rupture before the egg was opened. Figure 1 shows a bleb which could be seen through the inner shell membrane after the shell was removed from over the air space in the large end of the egg. The inner shell membrane was carefully peeled away before the photograph was taken. The egg was exposed to nitrogen for 32 hours and opened at 38 hours.

Figure 2 shows the blastodisk region of an egg exposed to nitrogen for 32 hours and opened after 38 hours of incubation. The border of the area pellucida is irregular, the area opaca is fragmented, and no development is seen.

The area opaca seen in Figure 3 is also fragmented, but less severely than the one in Figure 2. This embryo was exposed to nitrogen for 24 hours and examined after 38 hours of incubation. The worst fragmentation was commonly found at the anterior end of the area opaca.

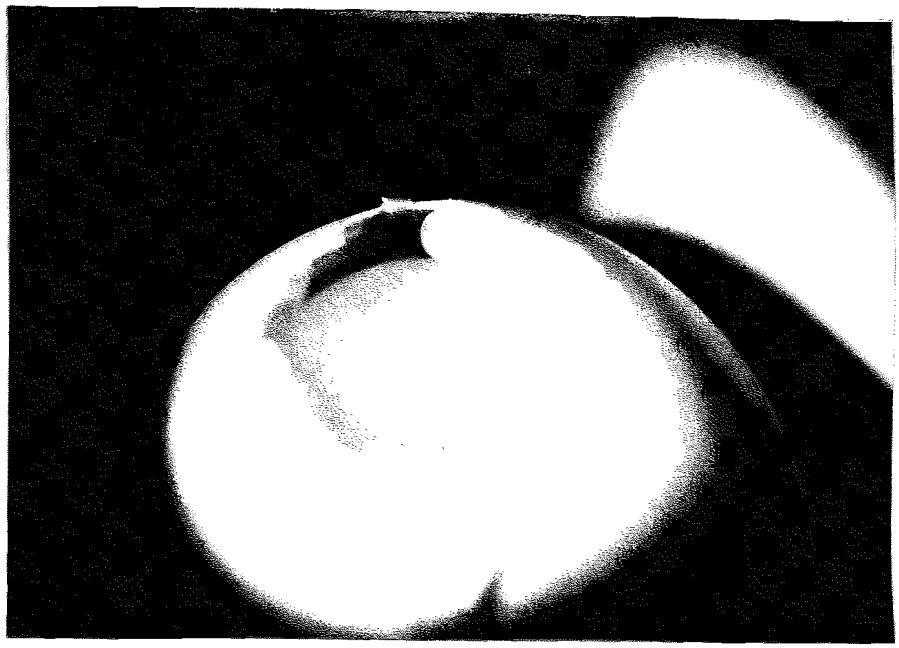

Figure 1. Egg incubated for 38 hours, exposed to 100% nitrogen for the first 32 hours of incubation. The shell and inner shell membrane have been removed from the large end of the egg. The weakened vitelline membrane has allowed the yolk to form a bleb.

Figure 2. 38 hour blastodisk exposed to 100% nitrogen for the first 32 hours of incubation. The area opaca is fragmented, and no embryonic development is seen.

As a result of hypoxia, embryonic tissues which normally come together and fuse fail to do so properly. Examples of this meeting and fusing process are in neural tube formation and body cavity closure. Whether caused directly by the lack of oxygen, or by the general retardation of development produced by hypoxia is not known, though evidence exists that both factors may be involved. The tissues may lose the ability to fuse after a certain period of time. The lack of oxygen might cause a direct interference with the energy requirements of the embryo. The term "phase-specificity" is used by Grabowski to indicate "that the structures which, at the moment of treatment, have the greatest respiratory activity are the structures which are most likely to be affected."[1]

Figure 4 shows a 38 hour embryo exposed to nitrogen for the first 16 hours of incubation. The embryo showed retarded development, but otherwise looked normal. The neural folds, instead of tapering gradually from the posterior end of the embryo to the point of dorsal fusion, lie well separated until just before fusion is to occur. This may be compared with Figure 5, which is a normal 38 hour embryo.

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[1] C. T. Grabowski, and J. A. Parr, "The Teratogenic Effects of Graded Doses of Hypoxia on the Chick Embryo," American Journal of Anatomy, CIII (November, 1958), 313-347.

Figure 3. 38 hour embryo exposed to 100% nitrogen for the first 24 hours of incubation. Area opaca is fragmented most severely at the anterior end. Hensen's node is visible. Development corresponds to that of a normal embryo of about 20 hours incubation.

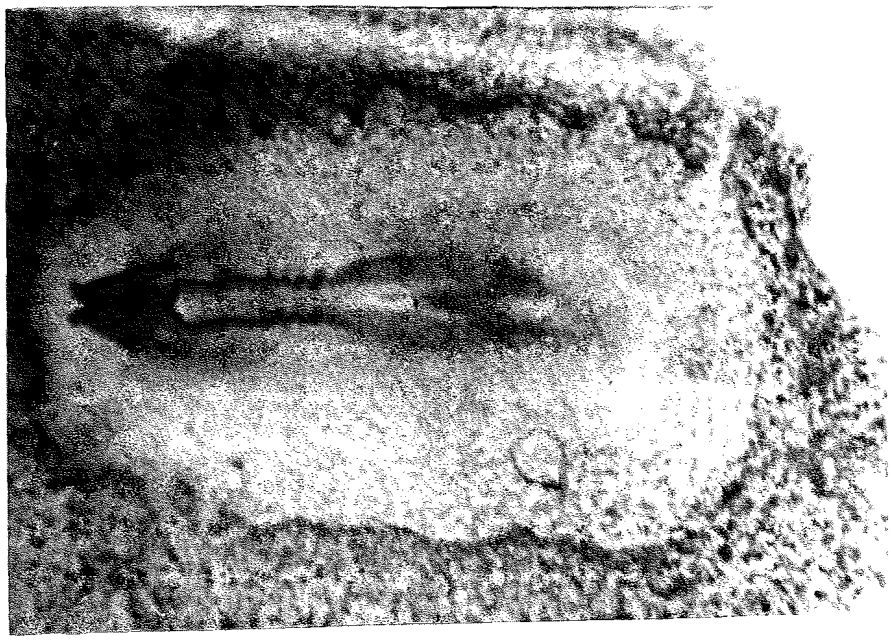

Figure 4. 38 hour embryo exposed to 100% nitrogen for the first 16 hours of incubation. This embryo looks normal, but is retarded. Development corresponds to that of a normal embryo of about 24 hours incubation.

Some parts of an embryo are more sensitive to hypoxia than are other parts. Evidence for this is the frequency with which abnormalities occur in the various parts of the embryo. Mid- and fore-brain regions of the neural tube, as well as the tail bud region are most sensitive to lowered oxygen tensions. Certain parts of the embryo are more capable than others of carrying on anaerobiosis. The head and tail bud regions depend almost entirely on aerobic metabolic mechanisms.[1] Lack of oxygen seems to interfer with the organizing centers in the embryo. Grabowski states "that many of the anomalies induced by hypoxia occur at progressively more posterior levels of the body as the age at the time of treatment increases."[2]

Many of the abnormalities described by both Grabowski and Nelsen were observed during the course of this investigation. Some were produced in the preliminary experiments. One of the more interesting abnormalities is acleiencephaly, which arises when the brain region fails to complete the tubulation process. Pseudoencephaly results from the acleiencephalic condition, or from weak fustion of the roof plate area of the brain. Cells of the lateral brain walls

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[1]O. E. Nelsen, "Hypoxia and Maldevelopment of Early Alarplate Tissue in Mid- and Fore-Brain Regions of the Chick Embryo," Growth, XXIV (December, 1960), 361-383.

[2]C. T. Grabowski, and J. A. Parr, "The Teratogenic Effects of Graded Doses of Hypoxia on the Chick Embryo," American Journal of Anatomy, CIII (November, 1958), 313-347.

undergo movements of eversion to form a bilateral tumor-like mass on the outside of the head. Eversion movements are greatest when acleiencephaly is slight.[1] Figure 6 shows a pseudoencephalic embryo of 10 days incubation with a normally appearing 10 day embryo. Both of these embryos were exposed to 100% nitrogen for the first 24 hours of incubation. Besides slight acleiencephaly, this embryo also had a short upper beak and bilateral microophthalmia.

Development of blood vessels in the pseudoencephalic tissue is also abnormal, according to the histological studies of Nelsen. He described "sinus-like structures which contact the surface where they ultimately rupture."[2] Figure 7 shows an embryo which was alive when removed from the egg after 7 days of incubation and exposure to 100% nitrogen for the first 24 hours of incubation. The amnion was filled with blood when the egg was opened. The embryo could not be seen until the amnion was removed.

Figures 7, 8, 9, and 10 all show embryos with acute acleiencephaly. In Figures 9 and 10, the body cavity has also failed to close ventrally. The entire posterior part of the embryo in Figure 8 has failed to develop.

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[1] O. E. Nelsen, "Hypoxia and Maldevelopment of Early Alarplate Tissue in Mid- and Fore-Brain Regions of the Chick Embryo," Growth, XXIV (December, 1960), 361-383.

[2] Ibid.

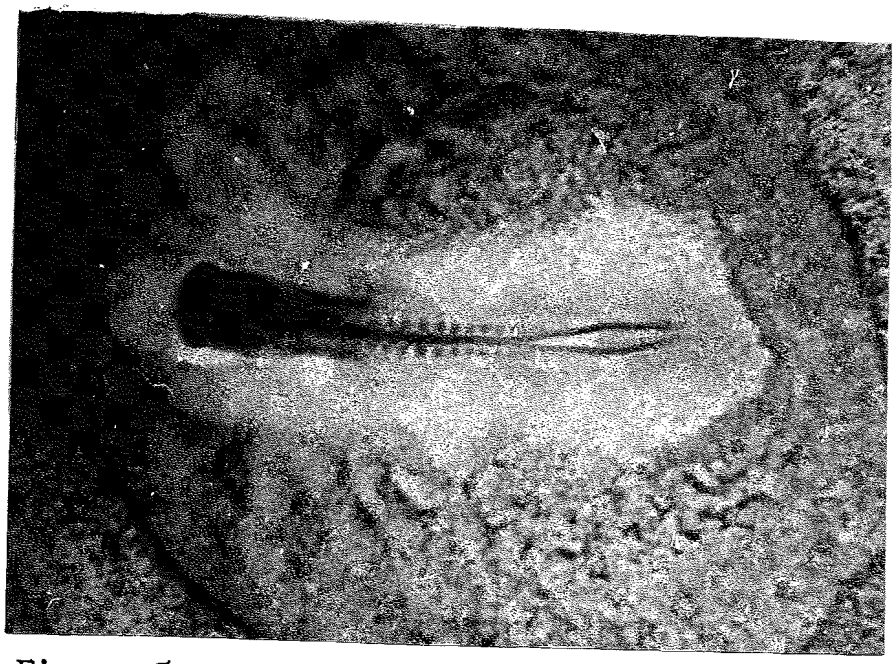

Figure 5. Normal 38 hour embryo. Embryo length = 4.4 mm. Width of area vasculosa = 7.0 mm.

Figure 6. 10 day embryos exposed to 100% nitrogen for the first 24 hours of incubation. The embryo on the left appears normal; the one on the right is slightly retarded, has a short upper beak, bilateral microphthalmia, and is pseudoencephalic. Note the tumor-like growth on the head.

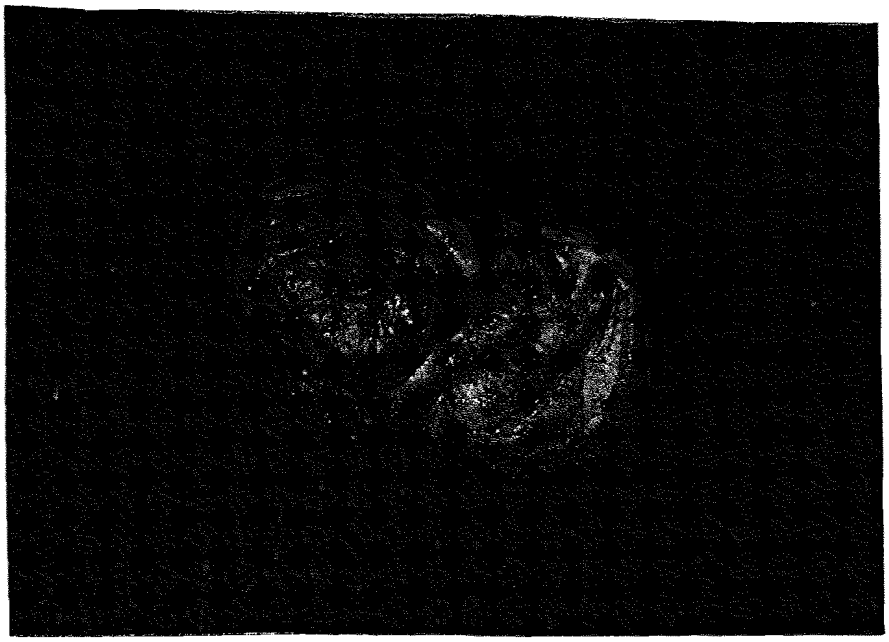

Figure 7. 7 day embryo exposed to 100% nitrogen for the first 24 hours of incubation. A hemorrhage caused the amniotic cavity to fill with blood. This embryo shows severe pseudoencephaly.

Figure 8. 7 day embryo exposed to 100% nitrogen for the first 16 hours of incubation. This embryo shows acute pseudoencephaly, and the entire posterior part of the embryo has failed to develop.

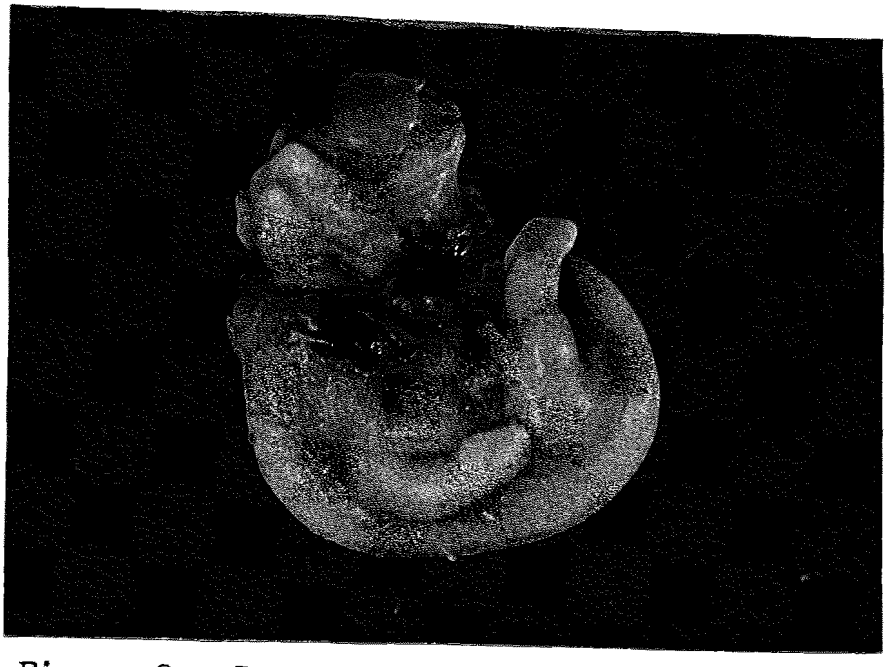

Figure 9. 7 day embryo exposed to compressed air for the first 16 hours of incubation. This embryo shows acute acleiencephaly, and incomplete closure of the body cavity.

Figure 10. 8 day embryo exposed to 100% nitrogen for the first 24 hours of incubation. The body cavity has failed to close ventrally, and the head shows moderate pseudoencephaly. The upper and lower beaks are visible, but no eyes have developed.

The anterior, posterior, or both ends of embryos subjected to hypoxia frequently exhibit delayed or abnormal development. The middle portion of the embryos show abnormalities less often. Figures 11 and 12 show embryos exposed to nitrogen for the first 16 hours of the total of 48 hours of incubation. The development of the head of the embryo in Figure 11 was greatly retarded and the anterior portion was absent. The rest of the embryo appeared normal but retarded. The embryo in Figure 12 had acleiencephaly; the walls of the neural tube in the head region did not fuse. The somites were not well formed, appearing less symmetrical than normal. Compare the somites to those in Figure 5.

It is difficult to asses the true meaning of abnormalities seen at the 38 hour examination time. It was not possible to tell by simple observation if an embryo such as the one in Figure 12 was alive. Degenerating embryos may mimic abnormal living embryos.

The implications of the abnormalities seen after 7 days of incubation are more readily apparent. The embryo shown in Figure 13 had no leg bud development, and the tail was reduced. The embryo in Figure 14 had a much reduced mesencephalon and abnormal tail development. Both of these 7 day embryos were exposed to 24 hours of 100% nitrogen beginning at the time of incubation, and both were alive (the heart was beating) when removed from the egg.

In Figure 15 are shown two embryos subjected to 24 hours

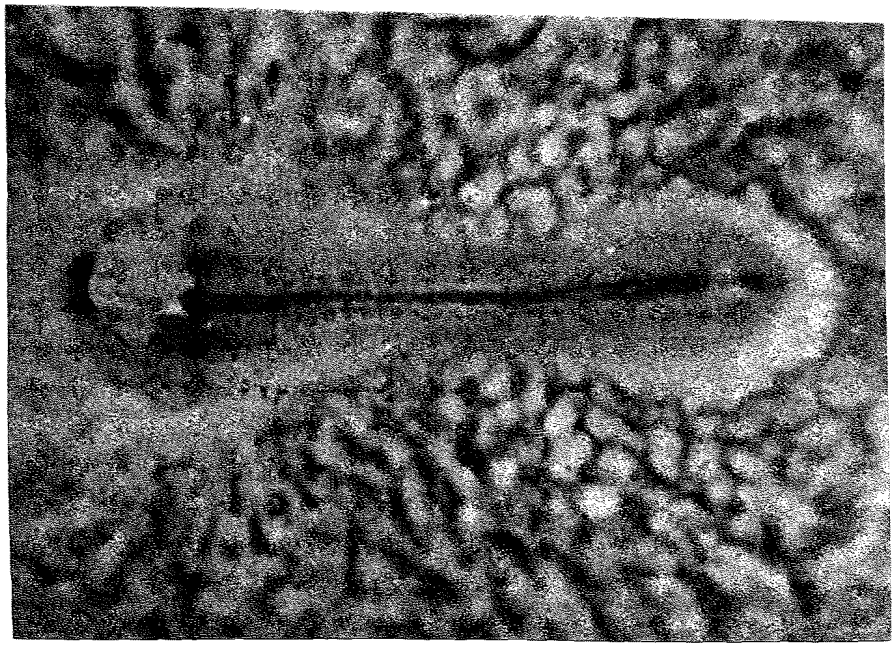

Figure 11. Embryo exposed to 100% nitrogen for the first 16 hours of incubation; total incubation time was 48 hours. The head has failed to develop, but the rest of the embryo appears normal.

Figure 12. Acleiencephaly in a 48 hour embryo exposed to 100% nitrogen for the first 16 hours of incubation. The embryo looks normal except for the open neural tube at the anterior end. Development is also retarded.

Figure 13. 7 day embryo exposed to nitrogen for the first 24 hours of incubation. The tail is reduced and no leg buds have developed.

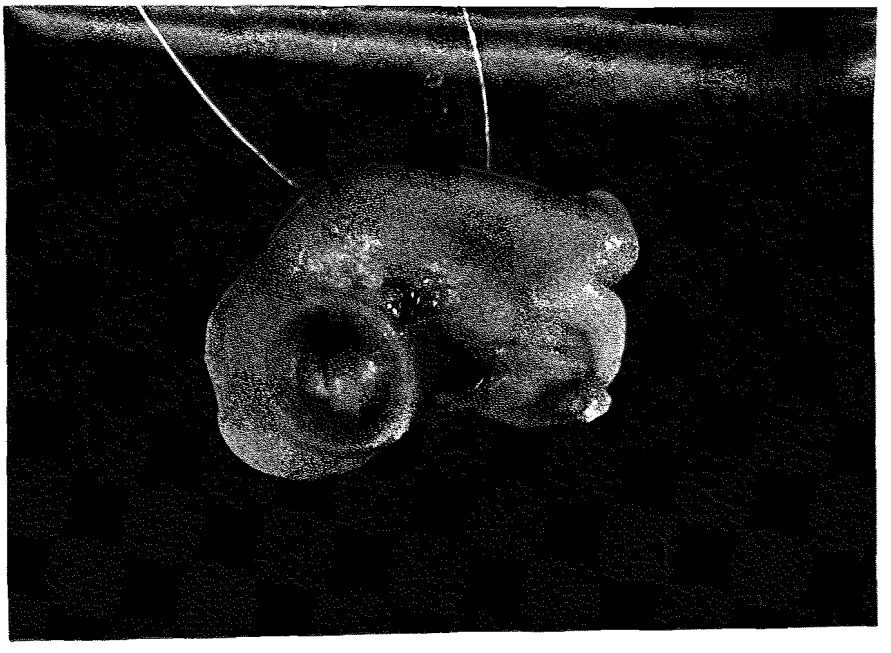

Figure 14. 7 day embryo exposed to nitrogen for the first 24 hours of incubation. The mesencephalon is much reduced and the tail bud is abnormal. The heart was beating when removed from the egg.

of 100% nitrogen immediately before being incubated. The eggs were kept at room temperature for 3 days prior to incubation. Figure 16 shows another view of the left embryo in Figure 15. The tail was absent, and no feet were formed.

The 8 day embryo in Figure 17 was subjected to 24 hours of 100% nitrogen beginning at the time of incubation. This embryo had unilateral anophthalmia and the tail was reduced to a small nib at the base of the spine.

Grabowski points out an interesting connection between hereditary and environmental factors in homozygous lethal Creeper fowl embryos which:

> ... can be detected after 54 hours of incubation by the anomalous development of the eyes and otocysts on the left (deep) side, lack of vitelline circulation, and a large anastomosis between the dorsal aorta and anterior cardinal veins.[1]

The abnormal circulation to the head causes hypoxia on the left side of the embryo. Tissues on the right side close to the shell receive oxygen by diffusion through the shell. This has been confirmed by incubating these eggs in an oxygen enriched environment. The vascular system still developed abnormally, but no other head abnormalities occurred.[2]

Elevated levels of oxygen in the incubating environment do not become highly toxic until about 96 hours of incubation

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[1] C. T. Grabowski, and J. A. Parr, "The Teratogenic Effects of Graded Doses of Hypoxia on the Chick Embryo," American Journal of Anatomy, CIII (November, 1958), 313-347.

[2] Ibid.

when the vascular system is developing. None of the embryos in this study were subjected to 100% oxygen for more than 24 hours, so most of the embryos appeared normal.

Carbon dioxide produced the most drastic retardation of embryogenesis. When the eggs were opened at 38 hours, no development was seen, except in the group exposed for 5 hours. It appears probable that the blastodisk cells were alive, since all embryos were developing when the eggs were opened after 7 days of incubation, though at a much reduced rate. At first it appeared that the embryos did not begin to develop until removed from the carbon dioxide and that this might be the reason for the smaller size of the embryos, since few gross abnormalities were seen. These embryos would have had a shorter effective incubation time, accounting for their smaller size if this was the case. However, the degree of retardation observed was even greater than would be expected if development started upon removal from the carbon dioxide. The preserving effects of carbon dioxide are reflected in the low mortality rates at the times at which the embryos were examined. It is doubtful that any of the embryos could have survived to the time of hatching. Figure 19 shows two 7 day embryos exposed to carbon dioxide for the first 24 hours of incubation and a normal 7 day embryo. Most of the embryos of this group exhibited varying degrees of microophthalmia, but otherwise appeared normal, except for their small size.

Figure 15. Both embryos exposed to 100% nitrogen for the 24 hours preceding incubation for 9 days in a normal environment. The embryo on the left has no tail, and no feet have formed. The right embryo is normal appearing, though retarded.

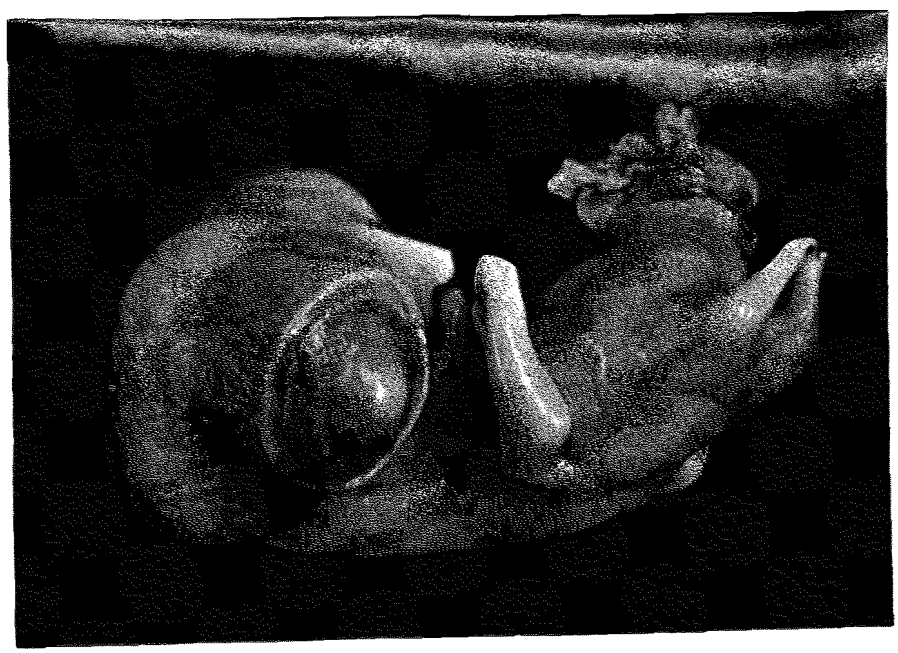

Figure 16. Right dorso-lateral view of the left embryo in Figure 15.

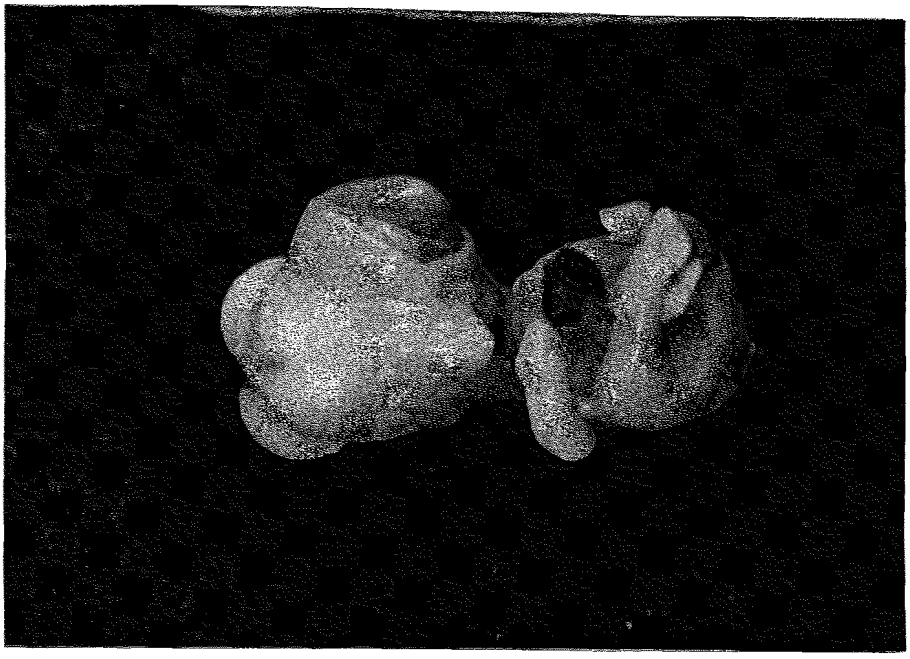

Figure 17. 8 day embryo exposed to 100% nitrogen for 24 hours beginning at the time of incubation. The Embryo has right anophthalmia and the tail is reduced to a nib at the base of the spine.

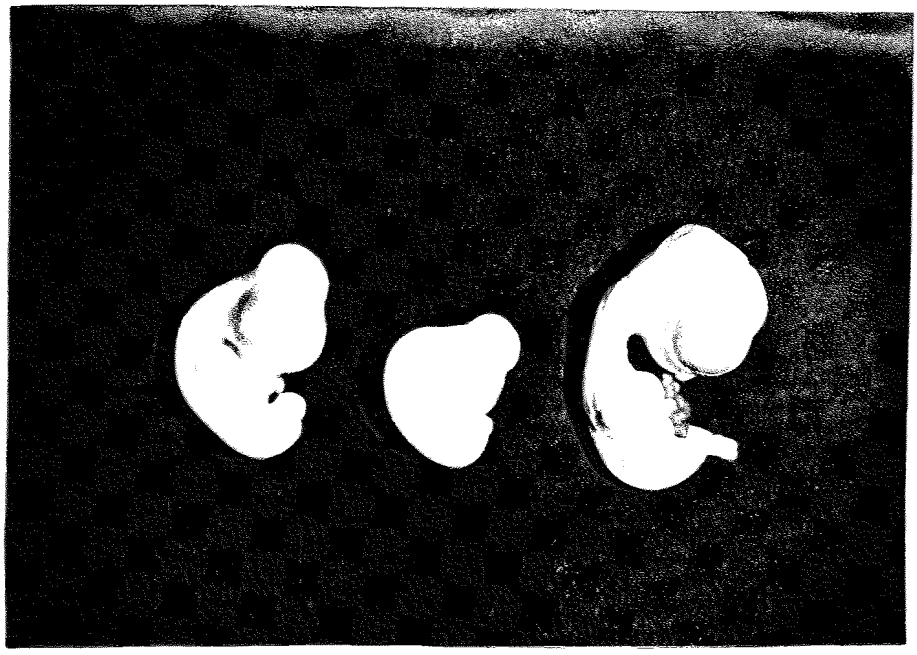

Figure 18. Two embryos on the left exposed to 100% carbon dioxide for the first 24 hours of incubation; total incubation time was 7 days. The right embryo is an untreated control of 7 days incubation. The two left embryos are retarded and show slight microphthalmia.

Since it is known that plastic films such as the saran formulation of Cryovac bags are semipermiable to various gases, it was desirable to know the permiability of the bags being used. A sample of the Cryovac plastic was sent to the W. R. Grace Company, Cryovac Division, P. O. Box 464, Duncan, South Carolina. A letter from Mr. N. D. Bornstein, Manager, Research & Analytical Services produced the following information on the transmission rates for a 1.0 mil film of Cryovac plastic type 930 standard:

Oxygen	152 cc/mil (24 hrs., m^2, atm.) @ 73° F
Carbon Dioxide	987 cc/mil (24 hrs., m^2, atm.) @ 73° F
Nitrogen	46 cc/mil (24 hrs., m^2, atm.) @ 73° F

The bags which were used had a mean thickness of 1.79 mils, which gives the following transmission rates:

Oxygen	84.9 cc/m^2 (24 hrs., atm.) @ 73° F
Carbon Dioxide	551.2 cc/m^2 (24 hrs., atm.) @ 73° F
Nitrogen	25.7 cc/m^2 (24 hrs., atm.) @ 73° F

The bags were 26 in. x 35 in. when layed flat, and had a surface area of approximately 1 m^2 when inflated and tied with string. The pressure within the bags was about 1 atmosphere, since the bags were left limp after inflation.

Further studies of the effects of anoxia produced by displacement of oxygen with nitrogen are needed. Eggs should be placed in Cryovac bags inflated with nitrogen, and then let stand at room temperature for a few hours

before being incubated to allow the gases to equilibrate within the egg. Soda lime should be put in the Cryovac bags to absorb excess carbon dioxide. Histological studies of early blastodisk differentiation may help elucidate the mechanisms of the production of malformations seen in later embryos.

CHAPTER IV

SUMMARY

Hypoxia is associated with malformations in developing chick embryos, and presumably in other species as well. The mode of action is thought to be at the molecular level by the poisoning of enzyme systems, or at the cellular level by the disruption of organizing centers. Retarded development is generally associated with malformations.

High levels of oxygen may be as detrimental to living tissues as levels below normal. The purpose of this experiment was to examine the effects of atmospheres of 100% oxygen (hyperoxia), 100% nitrogen and carbon dioxide (anoxia) on the early embryogenesis of the chick.

Fertilized eggs of White Leghorn hens were incubated for 5, 10, 16, and 24 hours in Cryovac plastic bags inflated with nitrogen, oxygen, or carbon dioxide, exposure to the gases beginning at the start of incubation. The eggs were removed from the bags at the specified time and returned to the incubator. Half of the eggs were examined after 38 hours of incubation, and the other half examined after 7 days.

One hundred per cent oxygen had little effect on the developing embryos. Nitrogen (anoxia) produced detrimental effects proportional to the length of exposure. The most effective range for the production of teratologies was 16-24 hours exposure to nitrogen. Carbon dioxide had a

preserving effect on the vitelline membrane, and subsequent embryonic development after removal from the gas was greatly retarded.

Further studies of the effects of anoxia produced by exposure to 100% nitrogen on the vitelline membrane, extraembryonic membranes, and neural tube formation in the early chick embryo using histological techniques are needed. Soda lime should be included in the Cryovac bags to absorb excess carbon dioxide.

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